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C-C Chemokine Receptor 4 Expression Defines a Major Subset of Circulating Nonintestinal Memory T Cells of Both Th1 and Th2 Potential

David P. Andrew, Nancy Ruffing, Chang H. Kim, Wenyan Miao, Heidi Heath, You Li, Kristine Murphy, James J. Campbell, Eugene C. Butcher, and Lijun Wu

CCR4, a chemokine receptor for macrophage-derived chemokine (MDC) and thymus and activation-regulated chemokine (TARC), has been implicated as a preferential marker for Th2 lymphocytes. Following in vitro polarization protocols, most Th2 lymphocytes express CCR4 and respond to its ligands TARC and MDC, whereas Th1 lymphocytes express CXC chemokine receptor 3 and CCR5 (but not CCR4). We show in this study that CCR4 is a major receptor for MDC and TARC on T lymphocytes, as anti-CCR4 mAbs significantly inhibit the migration of these cells to MDC and TARC. CCR4 is also highly expressed in most single-positive CD4⁺ lymphocytes and on a major fraction of blood nonintestinal (αvβ7⁺) memory CD4⁺ lymphocytes, including almost all skin memory CD4⁺ cells expressing the cutaneous lymphocyte Ag (CLA), but weakly or not expressed in other subsets in thymus and blood. Interestingly, major fractions of circulating CCR4⁺ memory CD4 lymphocytes coexpress the Th1-associated receptors CXCR chemokine receptor 3 and CCR5, suggesting a potential problem in using Th1 cells vs Th2 lymphocyte cells. Moreover, although production of Th2 cytokines in blood T cells is associated with CCR4⁺ CD4 lymphocytes, significant numbers of freshly isolated circulating CCR4⁺ memory CD4 lymphocytes (including both CLA⁻ and CLA⁺ fractions) readily express the Th1 cytokine IFN-γ after short-term stimulation. Our results are consistent with a role for CCR4 as a major trafficking receptor for systemic memory T cells, and indicate that the patterns and regulation of chemokine receptor expression in vivo are more complex than indicated by current in vitro models of Th1 vs Th2 cell generation.

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Chemokines are a large and growing family of 6- to 14-kDa heparin-binding proteins that mediate a wide range of biological functions (1–4). In various in vitro assays, chemokines support the chemotaxis or transendothelial migration of leukocytes, while in vivo injection (5) or overexpression of chemokines (6) results in leukocyte accumulation at the site of chemokine expression. Antagonists of chemokines and their signaling prevent leukocyte trafficking (7) and have beneficial effects in several acute and chronic inflammatory models (8, 9). Chemokines also modulate angiogenesis (10) and hemopoiesis (11) as well as T lymphocyte activation (12, 13), and several act as coreceptors with CD4 for entry of M-tropic and T-tropic HIV-1 (2).

Several subsets of CD4 lymphocytes can be defined based on their expression of adhesion molecules CD62L, cutaneous lymphocyte Ag (CLA), α4β7, and α5β1, and these various CD4 lymphocyte subsets traffic to different physiologic sites (14). For example, CLA⁺ CD4⁺ memory lymphocytes for cutaneous Ags and are thought to traffic to the skin (15), while the opposite subset of αvβ7⁺ memory CD4 lymphocytes traffics to intestinal sites (16). Leukocyte adhesion to endothelium involves several overlapping steps of rolling, activation, and arrest. Exposure of rolling leukocytes to factors expressed at the adhesion site activates leukocytes and up-regulates integrin-mediated adhesion. As a result of this interaction, leukocytes arrest on the endothelium (7, 17). Leukocyte activation and up-regulation of integrin molecules occur via a pertussis toxin-sensitive mechanism that is thought to involve chemokine receptors (7, 18). A growing body of evidence indicates that leukocyte subsets will also be defined by the expression of chemokine receptors required to traffic to various physiologic sites (19).

Several chemokine receptors are expressed on subsets of memory CD4 lymphocytes. For example, CXC chemokine receptor 3 (CXCR3), CCR2, and CCR5 (20–22) are all expressed on subsets of memory CD4 lymphocytes, while certain chemokines act selectively on naive T cells (23) or memory CD4 lymphocytes (24). Furthermore, several chemokines that interact with these receptors have been shown to be expressed in inflammatory sites (25) and lymph nodes (26). Within the memory CD4 lymphocyte population are subsets of effector T lymphocytes, Th1 and Th2, classified by the cytokines they secrete (27). Th1 lymphocytes secrete cytokines such as lymphotixin and IFN-γ to promote cellular immune responses, while Th2 lymphocytes release cytokines such as IL-4 and IL-5 to promote the humoral immunity and allergic responses (28). Recently, it has been shown that in vitro derived Th1/Th2 lymphocyte lines differentially express chemokine receptors. CXCR3 and CCR5 are selectively expressed by in vitro derived Th1 lymphocytes, while CCR4, CCR8, and CCR3 are expressed.
on Th2 lymphocytes (24, 29–33). Interestingly, the chemokines macrophage-derived chemokine (MDC) (stimulated T cell chem- 
moattractant protein-1) for CCR4 and IFN-γ-inducible protein-10 
for CXCR3 are induced by cytokines associated with Th1 and Th2 
environments, respectively (24, 34, 35). Furthermore, Imai et al. 
(33) showed that T cells attracted by thymus and activation-regu-
lated chemokine (TARC) and MDC predominantly produce Th2-
type cytokines, and that fractionated CCR4+ cells also preferential-
ly produce Th2-type cytokines.

In the studies reported in this work, by using specific anti-CCR4 
Abs, we confirm that CCR4 is differentially expressed on in vitro 
derived Th1/Th2 effector lymphocytes and demonstrate that CCR4 
is the major chemokine receptor on CD4 lymphocytes through 
which MDC and TARC act. Moreover, we define a large subset of 
memory CD4 lymphocytes that express CCR4, which comprise 
~50% of memory CD4+ cells, including nearly all CLA+ cells. A 
subset of CCR4+ cells coexpresses CXCR3 and CCR5 that are 
associated with in vitro derived Th1 cells. Although Th2 cytokine 
production is linked with expression of CCR4, CCR4+ memory T 
cells also contain non-Th2 cells expressing IFN-γ. Therefore, con-
trary to the preferential expression pattern of CXCR3, CCR5, and 
CCR4 on T cells that are generated in vitro in polarized conditions, 
CCR4 cannot specifically mark Th2 populations in vivo.

Materials and Methods

Construction of CCR4 stable transfectants and other 
transfectants

CCR4 cDNA was obtained by RT-PCR on RNA isolated from a basophilic 
cell line KG1a using a 5′-oligonucleotide primer and a 3′-oligonucleotide 
primer, which correspond to the CCR4 sequences and contain flanking 
HindIII and XhoI sites, respectively. The PCR fragment was subcloned into the 
HindIII and XhoI sites of pMRB101, in which the inserted gene ex-
pression was driven by a CMV promoter. The DNA was stably transfected 
into a murine pre-B lymphoma cell line (L1.2), as described (36–38). The 
cells expressing high levels of CCR4 were selected by serial dilution/sub-
cloning for their ability to chemotax to TARC and MDC. To enhance the 
receptor expression, the cells were treated at 106/ml with 5 mM butyric 
acid for 16–18 h before the experiments.

Other cell lines used included stable transfectants of the L1.2 murine 
pre-B lymphoma cells expressing CCR1–3, CCR5–9, CXCR1–CXCR5, as 
well as transfectants expressing various orphan G protein-coupled recep-
tors, including BONZO, BOB, LyGPR, APJ, RDC, GPR5, GPR9-6, and 
AF014958.

Purification of cell populations

Venous blood was collected from volunteer donors, and PBMC were iso-
lated by Ficol-Hypaque density-gradient centrifugation, as described (36). 
To isolate different subsets of leukocytes, human peripheral blood was 
collected in 10% (v/v) 0.1 M EDTA, layered onto 1-Step Polymorphs 
gradient (Accurate Chemicals, Westbury, NY), and centrifuged at 400 × g 
for 30 min at room temperature. Neutrophil and mononuclear cell layers 
were collected, resuspended in Dulbecco’s PBS without calcium and mag-
nesium (Life Technologies, Grand Island, NY), and centriﬁuged for 15 min 
at ~750 × g. RBC were lysed in the neutrophil fraction by resuspending 
the pellet in E-Lyse (Cardinal Associates, Santa Fe, NM) for 5 min on ice. 
Both cell fractions were washed twice with ice-cold Dulbecco’s PBS. The 
mononuclear cells were allowed to adhere to protein-coated plastic for 2–3 h, 
and then nonadherent cells were gently washed off the plate. CD4 cells 
were purified from mononuclear cells with the relevant Miltenyi beads 
using 20 μl of beads for 106 mononuclear cells in PBS/5% BSA/5 mM 
EDTA at 5 × 105 cells/ml for 30 min at 4°C. They were then spun down, 
resuspended in PBS/1% BSA/5 mM EDTA at 5 × 103 cells/ml, and passed 
over a VS column (Miltenyi Biotech, Auburn, CA) in a magnetic ﬁeld to 
remove nontagged cells. Cells were removed by forcing 20 ml of PBS/1% 
BSA/5 mM EDTA over the VS column, outside the magnetic ﬁeld.

Preparation of chronically activated Th1 and Th2 lymphocytes

As previously described (39), six-well Falcon plates were coated overnight 
with 10 μg/ml anti-CD28 and 2 μg/ml OKT3, and then washed twice with 
PBS. Umbilical cord blood CD4 lymphocytes (Poietic Systems, German-
town, MD) were cultured at 104–105 cells/ml in DMEM with 10% FCS and 
IL-2 (4 ng/ml), IL-12 (5 ng/ml) and anti-IL-4 (1 μg/ml) were used to direct 
to Th1, while IL-4 (5 ng/ml) and anti-IFN-γ (1 μg/ml) were used to direct 
to Th2. After 4–5 days, the activated Th1 and Th2 lymphocytes were 
restimulated once in DMEM and expanded for 4–7 days in DMEM with 10% 
FCS and IL-2 (1 ng/ml). Following this, the activated Th1 and Th2 lymph-
cytes were restimulated for 5 days with anti-CD28/OKT3 and cyto-
kines, as described above, with the addition of anti-CD95L (1 μg/ml) 
to prevent apoptosis. After 4–5 days, the Th1 and Th2 lymphocytes were 
was washed and then expanded again with IL-2 for 4–7 days. Activated Th1 
and Th2 lymphocytes were maintained in this way for a maximum of three 
cycles.

Generation of anti-CCR4 mAbs

C57/Black mice were immunized with 107 CCR4/L1.2 transfectants per 
mouse at 2- to 3-wk intervals for 6–12 times. For the ﬁrst three immuniza-
tions, the transfectants were pretreated with mitomycin C for 30 min 
before washing and injection to prevent tumor growth in the mice. Splen-
ens were harvested from mice 3–4 days after the last immunization, and cells 
were fused with the SP2/0 cell line, as described (40). Generally, 3000– 
5000 hybridomas were generated per fusion and the supernatants were 
tested for differential staining of CCR4 L1.2 transfectants and other che-
nomike receptor L1.2 transfectants. Of the 15 fusions performed, two fu-
sions gave rise to anti-CCR4 mAbs, IgG1 (isotype IgG1) and 2B10 (isotype 
IgG2a), respectively.

Other Abs and reagents

Anti-CD4, -CD8, -19, -49d, -56, -62L, -CLA, -CD45RA, and -CD45RO 
dye-linked mAbs for immunofluorescence studies were all obtained from 
PharMingen (San Diego, CA). OKT3, an anti-human CD3 mAb, was ob-
tained from American Type Culture Collection (ATCC, Manassas, VA), 
and anti-human CD28 mAb was purchased from Becton Dickinson (San 
Diego, CA). The majority of the anti-chemokine receptor mAbs were gen-
erated in LeukoSite (Cambridge, MA) and have the clone names ID9 
(CCR2), 7B11 (CCR3), 2D7 (CCR5), 11A9 (CCR6), and 1C6 (CXCR3).

Recombinant human chemokines were obtained from PeproTech (Rocky 
Hill, NJ) and R&D Systems (Minneapolis, MN). The human endothelial 
cell line ECV304 was purchased from ATCC. All cytokines were obtained 
from R&D Systems.

125I-labeled human TARC (125I-TARC) and MDC (125I-MDC) 
binding

125I-TARC and 125I-MDC were purchased from DuPont-NEP (Boston, MA) 
and Amersham (Arlington Heights, IL), respectively, and unlabeled 
chemokines were obtained from PeproTech or R&D Systems. Chemokine 
bindings to target cells were conducted using the following procedure: 
CCR4/L1.2 cells were washed and resuspended in binding buffer (50 mM 
HEPES, pH 7.5, 1 mM CaCl2, 5 mM MgCl2, and 0.5% BSA) at 107/ml for 
each binding reaction (in a final volume of 100 μl), 25 μl of cell suspension 
(2.5 × 105 cells) was mixed with 0.1 nM radiolabeled chemokine with 
or without an appropriate amount of anti-CCR4 mAb or an isotype-matched 
control mAb. Total binding was measured in the presence of radiolabeled che-
nomikes only, and nonspeciﬁc binding (background) was determined in the 
presence of 100 nM unlabeled chemokines. The reactions were incubated 
at room temperature for 45–60 min, and stopped by transferring the mix-
ture to Unifilter-96 GF/B (Packard) plates that were then washed two 
to three times with binding buffer containing 0.5 M NaCl. The plates were 
dried and MicroScint scintillation ﬂuid was added before counting. Each 
sample was assayed in duplicate.

Chemotaxis assay

The 3-μm-pore-diameter Transwell inserts were used either uncoated 
or coated with 2% gelatin for 2 h. Then 0.45 ml of DMEM with 5% FCS was 
placed in the lower wells of the chambers, and 2 × 105 ECV304 cells were 
decided to each gelatin-coated insert in 0.2 ml of DMEM/5% FCS. After 2 
days, the wells and inserts were washed twice with RPMI containing 0.5% 
BSA and 10 mM HEPES, and then chemokine was added to the lower well.

For chemotaxis of transfectant cells, no ECV304 cells were used to coat the 
plates. The cells under study were washed once in RPMI and resuspended 
at 4 × 103 cells/ml for Th1/Th2 lymphocytes and cell lines and at 105 
cells/ml for transfectants and resting CD4 lymphocytes in RPMI containing 
0.5% BSA and 10 mM HEPES. An aliquot of 200 μl of cell suspension 
(input of 8 × 105 cells and 2 × 106 cells, respectively) was added to each 
insert. After 2–4 h, the cells migrated to the lower well, and the plates were 
dried and MicroScint scintillation ﬂuid was added before counting. Each 
sample was assayed in duplicate.
be 25,000 cells for Th1/Th2 cells and 75,000 cells for resting CD4 lymphocytes, in which this number represents the cells in the lower well counted on the FACScan for 1 min. To study the phenotype of migrating cells, identical experiments with CD4 lymphocytes were performed with six-well plates using 24-mm-diameter inserts. Chemotaxis assays were identical with ECV304 migration assays, but fibronectin-coated inserts (10 μg/ml) were used. In all cases, the data points were the result of duplicate wells, with the mean value shown and the error bars representing the sample SD.

Intracellular cytokine staining

Mononuclear cells from human peripheral blood were prepared by centrifugation on Histopaque-1077 (Sigma, St. Louis, MO). Untouched CD4+ T cells (purity >97%) were isolated by depleting non-CD4+ T cells using a magnetic bead depletion method (Miltenyi Biotec). CD4+ T cells were stained with Abs to CLA, CD45RA, and CCR4, CD45RA- (naive) CCR4+ CLA-, CD45RA- (memory) CCR4+ CLA-, CD45RA- CCR4+ CLA+, and CD45RA- CCR4+ CLA+ cells were sorted using FACStar (Becton Dickinson, San Jose, CA). Sorted cells were activated for 5 h at 37°C with PMA (50 ng/ml) and ionomycin (1 μg/ml) in RPMI 1640 medium supplemented with penicillin/streptomycin, 10% FBS, 50 U/ml IL-2, and 1 μg/ml brefeldin A. Activated cells were fixed and permeabilized using Cytofix/Cytoperm solution (PharMingen) and stained with PE-conjugated isotype control Abs or mAbs to IL-2, IL-5, IFN-γ (from PharMingen), and IL-4 (Becton Dickinson). Data were analyzed on FACSCaliber using CellQuest program (Becton Dickinson).

Results

The mAbs 1G1 and 2B10 specifically react with CCR4/L1.2 transfectants

Two mAbs were raised in C57/Black mice to CCR4/L1.2 transfectants. Both mAbs reacted with the CCR4/L1.2 transfectants, but not with parental L1.2 cells. The specificity of these two mAbs for CCR4 was further extensively examined using a large panel of L1.2 transfectants. Neither 1G1 nor 2B10 cross-reacted with L1.2 transfectants expressing CCR1, CCR2, CCR3, CCR5, CCR6, CCR7, and CCR8 (shown in Fig. 1 for 1G1). They also did not cross-react with L1.2 transfectants expressing CXCR1-CXCR5, or orphan G protein-coupled receptors including BONZO, BOB, LyGPR, APJ, RDC, GPR5, GPR9-6, and AF014958 (not shown).

CCR4 is expressed on subsets of CD4 lymphocytes and a minor subset of CD8 lymphocytes in peripheral blood

Both 1G1 and 2B10 were used to examine the CCR4 expression on leukocyte subpopulations, and similar results were obtained with both mAbs. Anti-CCR4 mAb 1G1 was used in the FACS experiments described in this work. In initial two-color studies of peripheral blood, CCR4 was found to be expressed on a large subset (20–30%) of CD4 lymphocytes as well as on a smaller subset (3–5%) of CD8 lymphocytes (Fig. 2A). Although CCR4low and CCR4high subsets of CD4 lymphocytes were evident, CCR4 was expressed at low levels on CD8 lymphocytes. B lymphocytes, basophils, eosinophils, neutrophils, and NK cells did not express CCR4 (Fig. 2A and data not shown). As would be predicted from its expression on blood T lymphocytes, in examining a large panel of cell lines, CCR4 was found only on T cell lines (Peer and CEM cells, data not shown).

We then examined the subset of CD4 lymphocytes that express CCR4 in greater detail by three-color staining (Fig. 2B). The CD4

![FIGURE 2](https://www.jimmunol.org/)

**FIGURE 2.** CCR4 expression on peripheral blood leukocytes. A, Isotype-matched mAb control staining. B, Expression of CCR4 on freshly isolated PBMCs. A two-color staining protocol was used to assess expression of CCR4 and the cell markers, CD4 and CD8 (T cells), CD19 (B cells), and CD56 (NK cells). C, CCR4 expression on CD4+ T cells. A three-color staining protocol was used to assess the expression of CCR4 and the T cell subset markers, CD45RA (naive), CLA (skin homing), and αβ (gut homing), CD62L (peripheral lymph node homing), and CD49d on CD4+ T cells. The cells were analyzed by gating on CD4+ lymphocytes. CCR4 expression was shown in x-axis in all plots, and the cell marker expression was shown in y-axis and indicated in each quadrant. Quadrants were set according to the staining of control mAbs, and the staining was representative of multiple donors analyzed. All results shown were using mAb 1G1 (n = 3), and similar results were obtained when mAb 2B10 was used.
lymphocytes that expressed CCR4 were mostly of memory phenotype CD45RA^+CD45RO^-. As reported in our recent studies (41), memory CLA^-CD4 lymphocytes, which traffic to skin, were all CCR4^high. However, CCR4^high CLA^- and CCR4^low CLA^- CD4 lymphocytes were also evident. In contrast, the subset of memory "alpha beta" CD4 lymphocytes, which traffic to mucosal sites, expressed little or no CCR4. Moreover, the subset of memory CD4 lymphocytes that expressed the highest level of CCR4 was predominantly "alpha beta". Furthermore, the subset of memory CD4 lymphocytes that lacks "alpha beta" integrins ("alpha beta", "alpha gamma") all expressed high levels of CCR4 (Fig. 2B). CD62L was expressed on both CCR4^+ and CCR4^- subsets of memory CD4 lymphocytes.

To further correlate the expression of CCR4 on CD4 lymphocytes with its function, we examined the subset of CD4 lymphocytes that chemotax to MDC by FACS. We used the anti-CCR4 mAb 1G1 in this experiment, as binding of 2B10 to CD4 lymphocytes was inhibited by MDC (data not shown). CCR4^+ CD4 lymphocytes were enriched in the population of CD4 lymphocytes that chemotax to MDC (2.6-fold). We also saw an enrichment for CLA^- (4.5-fold) and CD45RA^- memory CD4 lymphocytes (2.9-fold) in the migrated population. Among CCR4^+ memory CD4 lymphocytes, the majority of the migration to MDC was in the CLA^-CCR4^+ subset rather than the CLA^-CCR4^- subset (4.5 vs 1.6). Finally, in examining the chemotaxis of 16 cell lines to MDC and TARC, we found that only the two cell lines that expressed CCR4 chemotaxed to MDC and TARC (data not shown). Therefore, expression of CCR4 on T lymphocytes and functional response to MDC/TARC correlate.

**CCR4 is expressed on a subset of mature single-positive (SP) CD4^+ thymocytes**

Because CCR4 transcripts are present in thymus, we examined the expression of CCR4 on thymocytes. CCR4 was expressed on a small subset (14%) of thymocytes (Fig. 3A). The expression of CD3 on all CCR4^+ thymocytes indicates that CCR4 is up-regulated during thymocyte maturation. Another chemokine receptor, CXCR4, was expressed on immature thymocytes, while CCR9 was expressed at all stages of T cell development. Due to the expression of CCR4 on mature thymocytes, we analyzed expression of this chemokine receptor in immature double-negative CD4^-CD8^- and double-positive CD4^+CD8^- lymphocytes as well as mature SP CD4 and CD8 lymphocytes (Fig. 3B). As expected, CCR4 expression was restricted to mature SP lymphocytes and was mainly expressed on SP CD4 lymphocytes. The majority of SP CD4 lymphocytes express CCR4, but a small subset of CCR4^-CD4^+ SP lymphocytes was also evident.

**mAbs 1G1 and 2B10 block the binding of MDC and TARC to CCR4**

The ability of the mAbs 1G1 and 2B10 to inhibit the binding of TARC (Fig. 4A) and MDC (Fig. 4B) to CCR4 was examined. Both mAbs blocked the 125I-TARC and 125I-MDC binding to CCR4/L1.2 transfectants, while the isotype-matched control mAbs had no significant effect. Furthermore, both 1G1 and 2B10 effectively blocked the TARC- and MDC-mediated chemotaxis in CCR4/L1.2 transfectants (Fig. 4A, C and D). Of greater interest, 2B10 and to some extent 1G1 significantly inhibited chemotaxis of resting CD4 lymphocytes (Fig. 5A) and Th2 lymphocyte (Fig. 5B) to TARC and MDC. Both mAbs had no effect on the chemotaxis of CD4 lymphocytes to RANTES.

**Activation of naive T cells in Th2 polarization condition is required for CCR4 expression on most T cells**

Although umbilical CD4 lymphocytes did not express CCR4 (data not shown), chronic activation of these cells in the presence of IL-4 to generate Th2 lymphocytes resulted in CCR4 expression (Fig. 6), while chronic activation with IL-12 to generate Th1 lymphocytes did not up-regulate CCR4 expression. As expected, CXCR3 was up-regulated on Th1 lymphocytes, while "alpha beta", an integrin used in lymphocyte trafficking to mucosal sites, and chemokine receptor CCR2 were up-regulated on both Th1 and Th2 lymphocytes (Fig. 6).

Activation of T lymphocytes with anti-CD3 mAb resulted in transient down-regulation of CCR4 over 2–3 days, with expression recovering upon cessation of TCR cross-linking and culture in the presence of IL-2 (data not shown). This is similar to what was
observed for CCR5, CCR6, and CXCR3. The CCR4 expression peaks between 4 and 8 days after activation, and declines thereafter.

**CCR4 is coexpressed with Th1-associated chemokine receptors CCR5 and CXCR3**

The expression of CCR4 on CD4 lymphocytes in relation to other chemokine receptors known to be expressed on subsets of memory CD4 lymphocytes was examined in three-color experiments (Fig. 7). CCR4 was found on both positive and negative subsets of CCR2 and CCR6 CD4 lymphocytes. Surprisingly, given the association of CCR5 and CXCR3 expression with Th1 phenotype by in vitro studies, a significant population of CCR5+CCR4+ and CXCR3+CCR4+ CD4 lymphocytes was observed in these experiments.

**Production of Th1 and Th2 cytokines by CCR4+ CLA−, CCR4+ CLA−, and CCR4− memory CD4 lymphocytes**

As CCR4 is coexpressed on memory CD4 lymphocytes with Th1-associated chemokine receptors, we next examined the cytokines produced by activated subsets of memory CD4 lymphocytes defined by expression of CCR4 and CLA. As expected, naive and memory CD4 lymphocytes secreted IL-2 on stimulation, while production of IL-4, IL-5, and IFN-γ was restricted to the memory CD4 lymphocytes (Fig. 8A). Production of IFN-γ was associated with the CCR4+ memory CD4 lymphocytes, with 51% of CCR4+ CLA− CD4 memory cells producing IFN-γ on activation, while only 17% of the CCR4+ CLA− and 10% of the CCR4+ CLA− memory CD4 lymphocytes produced this cytokine. Similarly, production of Th2 cytokines IL-4 and IL-5 was positively associated with CCR4 expression on CD4 lymphocytes, with the CLA− CCR4+ memory CD4 lymphocytes being the major producers of these two cytokines. However, while Th2 and Th1 cytokine production was positively and negatively associated with CCR4 expression, respectively, expression of IFN-γ was observed in significant (5–30%) subsets of activated CCR4+ memory CD4 lymphocytes, and IL-4 production was also observed in a subset (up to 3%) of freshly isolated CCR4+ memory CD4 lymphocytes on activation. Studies of additional normal donors for IL-2, IL-4, and IFN-γ production were also performed and similar results were observed (Fig. 8B).

To increase the percentage of cells producing Th2 cytokines, sorted CCR4+ and CCR4− memory CD4 lymphocytes were activated with anti-CD3 and expanded in IL-2 before performing activation and intracellular staining. Larger percentages of cells produced IL-4, IL-5, and IL-13, and similar results were obtained for CCR4+/CCR4− CD4 lymphocyte subset production of these cytokines: IL-4 (2.4%/12.5%), IL-5 (2.8%/14%), IL-13 (2%/24%), and IFN-γ (81%/24%).

Finally, we sorted CCR4+/−, CLA−/−, CD45RA−/− CD4 T cell subsets and stimulated them with anti-CD28 and anti-CD3 in nonpolarizing culture condition (without added exogenous IL-4 or IL-12 or other neutralizing Abs) for 4 days and then cultured them in IL-2 for 3 days (the first activation cycle). Half the cultured cells were examined for intracellular cytokine analysis, and the other half were cultured again (the second activation cycle). The second activation cycle contained not only more T cells producing IL-4, but also included more T cells producing IFN-γ than fresh CD4 T cells (Fig. 9). This is also the case for the CCR4− CD4 T cells. These combined data suggest that CCR4− CD4 T cells have potentials to become Th1 as well as to become Th2 cells.

**Discussion**

We have shown in this study that CCR4 is expressed on a subset of memory CD4+ lymphocytes, a very small subset of CD8+ lymphocytes, and thymocytes. Moreover, we have demonstrated that CCR4 is the major memory T lymphocyte trafficking receptor for
CCR4 EXPRESSION AND FUNCTION ON LEUKOCYTE SUBSETS

MDC and TARC. Finally, we provide evidence to suggest that despite its preferential expression on in vitro derived Th2 cells, CCR4+ memory T cell subset also contains non-Th2 populations in vivo.

Recently, TARC was reported to bind to CCR8 (42). However, in our hands and in other reports (43), TARC and MDC mediated only chemotaxis of CCR4 transfectants and not other chemokine receptor transfectants, including CCR8. A screening of a panel of cell lines in this study indicates that only CCR4+ cell lines chemotax to MDC and TARC. In addition, the majority of chemotaxis of both resting CD4+ cells and chronically activated Th2 lymphocytes to MDC and TARC can be blocked by anti-CCR4 mAbs. We also found that the subset of CD4 lymphocytes that migrated to MDC is enriched for CCR4+ expressing cells. These combined results suggest that MDC and TARC predominantly use CCR4 on T cells in mediating chemotaxis of CD4 lymphocytes.

Our studies demonstrate that CCR4 is expressed on a large subset of the SP CD4+ thymocytes that express high levels of TCR, suggesting CCR4 expression is induced on thymocytes at a late stage of T cell development. The restricted expression of CCR4 on SP CD4+ thymocytes correlates with the chemotaxis of this subset of thymocytes to MDC and further demonstrates that expression of CCR4 and chemotaxis to MDC correlate (44). TARC and MDC are also reported to be expressed in the thymus (45, 46) and by APCs (24, 47). It is possible that TARC and MDC are expressed on macrophages, dendritic cells, and epithelial cells in the thymus and attract mature CCR4+ CD4 SP thymocytes to modulate positive and negative selection during T cell development. Interestingly, CD45RA naive T lymphocytes in the periphery do not express CCR4. Therefore, on exit of SP CD4 thymocytes from the thymus to the periphery, CCR4 expression must be down-regulated. Alternatively, those SP CD4 thymocytes that do not express CCR4 in the thymus may represent fully mature thymocytes about to exit the thymus.

The subset of memory CD4 lymphocytes that express CCR4 encompasses nearly all CD4 lymphocytes that express CLA, but not α4β7. Indeed, nearly all CLA+ α4β7− memory CD4 lymphocytes express high levels of CCR4. However, most importantly, CCR4 is expressed at high levels by a subset of CLA+ α4β7− cells, suggesting that CCR4 may mediate other functions in addition to its proposed role in trafficking of CLA+ memory CD4 lymphocytes to skin. It was shown previously that CLA was expressed by T cells responsible for the Th2 cytokine production in individuals with skin allergy (48, 49). Nevertheless, in individuals with allergy at nonskin sites, Th2 lymphocytes were also found in the memory CLA+ CD4 subset. The expression of MDC by APCs, including activated B cells and the induction of MDC as well as CCR4 on T lymphocytes by Th2 cytokines (21, 32), indicates that CCR4 is possibly also involved in local movement of memory CD4 lymphocytes in lymphoid tissue toward dendritic cells and B cells. Finally, the expression of CCR4 on both CD62L+ and CD62L− memory CD4 lymphocytes indicates that the memory CCR4+ CD4 subset includes both effector CD62L− memory CD4 lymphocytes that are thought to traffic principally to inflammatory sites as well as resting memory CD62L+ CD4 lymphocytes that can most likely also traffic to secondary lymphoid organs such as peripheral lymph nodes.
It was previously shown that MDC and TARC specifically act on in vitro derived effector Th2 lymphocytes, while CCR4 transcripts are differentially expressed by in vitro derived Th1/Th2 lymphocytes (24, 30). Consistent with these observations, we and Imai et al. (33) found that CCR4 protein is preferentially expressed on the surface of in vitro derived Th2 cells. However, if CCR4 were a specific marker for Th2 cells, one would have predicted that CCR4 would be expressed by a very small subset of memory CD4 cells, as effector Th2 cells represent only a small subset of peripheral blood CD4+ cells. Therefore, the demonstration in this study that CCR4 is expressed on a large subset of circulating memory CD4 lymphocytes was surprising. As MDC blocks all staining of leukocytes by the anti-CCR4 mAb 2B10 and both our anti-CCR4 mAbs stain identical subsets (data not shown), we are confident that our results represent the distribution of CCR4 on leukocytes. Imai et al. also showed recently that, using an independent anti-CCR4 mAb, CCR4 was detected on ~20% CD4+ T cells (33), although the intensity of their staining was weaker than what we obtained using both of our mAbs.

Our experiments examining coexpression of CCR4 with other chemokine receptors on CD4 lymphocytes also gave unexpected results. We found that CCR4 was expressed on a significant subset of CD4 lymphocytes that also express chemokine receptor CCR5 and CXCR3, both of which are selectively expressed on in vitro derived effector Th1 lymphocytes. More importantly, our data suggest that although CCR4 behaves like a specific Th2 marker on in vitro derived Th2 lymphocytes, it does not specifically mark the Th2 population in circulating blood, and in this sense, neither do CCR5 and CXCR3 specifically mark the Th1 population. CCR4+ CD4 lymphocytes may represent a larger subset of memory CD4 cells, within which are contained the majority of Th2 lymphocytes. Alternatively, the culture conditions used to generate in vitro derived Th2 lymphocytes may induce expression of CCR4. In further studies, we directly examined the cytokines produced by memory CD4 lymphocytes from blood sorted for expression of CCR4 and CLA. Although IL-4-producing cells were more frequent in the CCR4+ than CCR4− subsets, there tended to be a greater frequency of IFN-γ-producing Th1 cells among the CCR4− subset. Furthermore, only a small subset of memory CCR4+ CD4 lymphocytes produces Th2 cytokines on activation. When freshly sorted CCR4+ and CCR4− CD4 lymphocytes were activated and expanded before performing intracellular cytokine staining, the Th2 correlation was still observed. It is possible that in certain chronic inflammation conditions, the linkage of CCR4 and Th2 phenotype may increase depending on cytokine expression profiles of tissue microenvironment. Further studies in patients with Th2 inflammatory diseases, such as atopic dermatitis and asthma, are underway to investigate this.

**FIGURE 7.** CCR4 is coexpressed with Th1-associated chemokine receptors CCR5 and CXCR3. Mononuclear cells were stained in a three-color study with 1G1, followed by F(ab′)2 anti-mouse IgG FITC. CD4 lymphocytes were marked with anti-CD4 tricolor, while subsets of cells and anti-CCR6, anti-CCR5, anti-CCR2, and anti-CXCR3 mAbs linked to PE were used to study the coexpression of these chemokine receptors with CCR4. The profiles shown represent the coexpression of CCR2, CCR5 and CXCR3 on CD4 lymphocytes, and gated on CD4+ cells. The experiment was performed twice with similar results.

**FIGURE 8.** Expression of effector cytokines by memory T cell subsets defined by expression of CCR4 and CLA expression. Peripheral blood CD4+ T cell subsets were isolated, activated for 5 h with PMA and ionomycin, and analyzed for cytokine expression, as described in Materials and Methods. One representative set of data from three independent experiments is shown (A). Data from six different donors are plotted to show trends and individual variability of IL-2, IL-4, and IFN-γ expression in memory T cell subsets (B). *, Indicates significant difference from CCR4− CD4 T cells (p < 0.02, Student’s t test).
and CCR4 receptor through which TARC and MDC act on memory CD4 lymphocytes. Chemotaxis of CD4 lymphocytes to MDC and a small subset of mature SP CD4 of subset-specific homing to particular sites such as skin. CXCR3, and CCR5) can be independently controlled and regulated. A tight linkage. Most importantly, our results indicate that this linkage is malleable, and suggest that effector cytokine production at all stages of memory effector T cell differentiation, very likely reflecting the different influence and refinement of local immune responses as a function of tissues, microenvironment, and cytokine milieu.

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References


